SCIENTIFIC REPORTS

Unpublished data should not be cited without permission of the authors.

ERRATA

Page	Column & Line	For	Read
	Director's review 5	departmentments	departments
	Director's review 41	studens	students
1	6	bacteripphages	bacteriophages
1	28	with o	with σ
1	37	MgCl2	$MgCl_2$
1	39	o-factor	σ-factor
1	41	o release	σ release
2	3	CPT	CTP
2	12	pppGpRyp	pppGpPyp
2	16	in vivo	*in vivo*
2	33	refamycin	rifamycin
2	37	de novo	*de novo*
3	11	purified enzyme preparations as vegetative cells are	omit
4	3	conincide	coincide
5	19	DEAE cellulose	DEAE-cellulose
5	24	DNA cellulose	DNA-cellulose
5	29	whereas,	whereas
5	52	5' end	5'-end
6	7, 9 & 10	(^{3}H)poly(dG)	^{3}H-poly(dG)
18	15	simplexvirus	simplex virus
19	35	coruse	course
24	50	plasmacell	plasma cell
26	19	agtigen	antigen
28	Photo. legend 2	agarrose	agarose
28	Photo. legend 6	with Comassie-Brilliant Blue G250. Anode is right.	omit
29	45 & 47	interval	intervals
30	5	IgG_2a	IgG_{2a}
34	Fig. 1-a. legend 4	left	right
40	18	perfermed	performed
40	28	antibody-absorptin	antibody-absorption
45	20	Misistry	Ministry
49	28	througn	through
54	38	there	these
54	43	any	many
60	Table 1.	sensitizatinn	sensitization
62	1	the human being	in human being
71	5	wether	whether
72	14	*E. Coli*	*E. coli*
77	42	lymohoid	lymphoid
78	21	dendent	dependent
80	Table	<79	>79
80	Table	Fhrlich	Ehrlich
92	left 18	ases	cases
93	right 24	al ergy	allergy
93	right 42	preqaration	preparation
93	right 48	reserrch	research
94	left 28	Aaal.	Appl.
96	right 16	spmples	samples
96	right 39	The Nucelic Acids	The Nucleic Acids

99	To the personnel of Dept. of Virology, add Postgraduate Student: Hisashi Ogura (小 倉 寿)

DEPARTMENT OF MOLECULAR IMMUNOLOGY

GENERAL SUMMARY

When the Department of Molecular Immunology started in 1967, one project, the study of plasmacytoma, began. It included 2 research projects, 1) immunochemical study of myeloma proteins and 2) oncogenic study of plasmacytoma in mice. The former is a chemical static aspect and the later is a biological dynamic aspect. When Dr. M. Takahashi joined the Department as an Assistant Professor in 1969, two other fields of reseach were added, 3) cytological study and 4) functional study of complement.

From these aspects we have been intending to clarify the relationship between immunity and cancer, 2 unknown objects.

Plasmacytoma is a special tumor, as a tumor of an immunological competent cell. We formerly believed that immunity was effective against cancer when the cooperation of immunological competent cells was well performed. The developmental process of plasmacytoma includes within itself a discrepancy between immunity and cancer. The study revealed that immunity was a real complex of independency and cooperation. Plasmacytoma developed in mice which responded with high humoral immunity and low cellular immunity against antigens injected. However, myeloma protein having antibody activity to the antigen injected was never obtained.

Antibody activity to the dinitrophenyl group which is unrelated to the injected antigen was found in 2 myeloma proteins obtained in our labolatory.

General decrease of immunity by aging was a project of the present study, refering to independncy, cooperation and inhibition of immunocompetent cells (see O. Daimaru's abstract).

Another project that developed from the study of M-component with antibody activity was the discovery of an antigen, lipopolysaccharide (LPS) of *E. coli* 055, which produced M-component with antibody activity, after intraperitoneal injection into BALB/c mice (see S. Sakai's abstract). Since the genetic background of mouse strains and antigenic determinant of LPS are clear and the appearance of M-component with antibody activity after the injection of the antigen was 100%, this finding will contribute much to present immunology.

One branch that grew from the immunochemical field was quantification of serum components by single radial immunodiffusion (SRD). In the case of human serum, we are continuing the measurement of 30 components in patients sera using techniques of microscale SRD and of lyophilized standard control serum with excellent technical help of Mrs. K. Okamoto and Miss K. Horiuchi and with a supply of specific antiserum to human serum components from the Japan Hoechst Company.

The accumulated data about serum proteins have been analysed with the electronic computer of Kanazawa University and

of Okayama University with the cooperation of Mr. M. Shako, Computer Center, Kanazawa University and Mr. H. Osaki, College of Technology, Okayama University. This research aimed at the diagnosis of cancer and the determination of application for immunotheraphy to cancer by serum protein analysis (see S. Migita's abstract).

In the case of mouse serum, genetic differences of immunoglobulins and components of complement were revealed after the success of preparing specific antisera to immunoglobulin subclasses.

The immunizing antigens were obtained from M-components of myeloma mice developed in our labolatory and from other components purified. Normal mouse standard serum was prepared in the same way as human control serum for control of mouse sera.

The finding that the level of immunoglobulin G subclass was genetically linked to the H-2 type of the mouse was unique and will be published soon (see S. Sakai's abstract).

The measurement of components of complement in the sera of human and of mouse revealed cooperative changes in acute phase proteins and either the classical pathway or the alternate pathway of complement in cases of cancer or inflamation.

From the cytological study, Ohno revealed the changes in the cytoplasmic membrane of the mouse myeloma cell during sucessive transplantation and its relation to immunoglobulin production of the cell (see S. Ohno's publicatin in 1975 and abstract).

Tokuyama carried out the study on the immune reaction against self-spleen-cells treated with trinitrobenzensulfonate (see H. Tokuyama's publication in 1975 and abstract).

The injection of isologous cells that were modified with trinitrophenyl group will be a model of immunity against cancer or of autoimmune disease, because the positive mixed lymphocyte reaction against isologous cells was observed in these experiments.

Takahashi has worked on the functions of complement especially C3 and C9, and on protein structural study of C3. His cytological study comes from the electron microscopic analysis using peroxidase labelled antibody technique. He revealed the subcellular organelle and the specific cell related to the synthesis of C3, $\beta 2$ microglobulin and myeloma protein (see M. Takahashi's publication).

These studies are focused to the function of C3 receptor of B lymphocyte at present. His experiments revealed that the B lymphocyte has a surface immunoglobulin and binds to corresponding antigen and early components of complement in the classical pathway on the cell membrane; then the release of the antibody-antigen-complement complex from the C3 receptor is a key for to the start of differentiation from B lymphocyte to plasmacell. He also revealed that the release was conducted with excess binding of C3b on the C3 receptor and the excess C3b comes from the resultant activation of the alternate pathway. Since M. Takahashi, S. Ohno and K. Yamamoto are ab-

sent in this Department at present and are each working in outstanding institutes of research in the U. S. A. and in Sweden, the activity of the Department at the present time is limited. We owe much to Prof. T. Saito for the participation of S. Sakai in cooperating with the work of our Department.

We believe that the activity of our Department with cooperation of the others will make progress, especially after 3 persons will join our Department in the near future.

This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan, a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan, a Grant-in-Aid for Cancer Research from Ministry of Health and Welfare, Japan and The Naito Research Grant.

ABSTRACT

(12) **Murine plasma cell tumor occurrence along with aging process: Evidence for T cell depression but not B cell depression.**
O. Daimaru, S. Natsuume-Sakai and S. Migita

It has been well documented that the immune function declined in the aging process, and this decline was mainly due to the decrease in T cell function. This depression may be a cause of deficiency of immune survaillance against tumor growth or autoimmune disease. We have been examing the developmental process of plasma cell tumors induced in BALB/c mice. It was found that the T cell function decreased in mice injected with Freund complete adjuvant (FCA) and plasmacytoma developed from the group with the depressed T cell function. In the present project, we examined the changes in the B cell function with the aging process and the changes after the treatment with FCA. Humoral immune response in mice was analysed using sheep red blood cells (SRBC) as an antigen.

Mice were injected intraperitoneally with 10^9 SRBC, and the number of plaque forming cells (PFC) per spleen was assayed by Counningham method 5 days after the injection. In normal BALB/c, peak response was obtained at the age of 2-3 months and then the response was reduced. At 16 months, PFC response was only 1/6 of the peak response.

It is well established that B stem cells exist in bone marrow.

Bone marrow cells (10^8) were transfered intravenously with thymocytes (10^8) obtained from young mice into 700R-irradiated syngeneic mice, followed by injection of 10^9SRBC. The highest response was confirmed with transfer of bone marrow cells which were obtained from mice within 2-3 months of age and the response, which was transfered with bone marrow cells from those of more advanced age, was reduced. From these results, it can be suggested that B cell function of the aged mice is depressed either at the level of the precursor or the stem cell.

Experiments were carried out with the mice, which were injected 3 times with 1 mg bovine-γ globulin (BGG) mixed with 0.5 ml FCA at 2 months intervals using as treatment for plas-

macytoma induction. The number of natural PFC to SRBC after one injection of BGG was 4 times greater than for normal mice, but the PFC response in those that were subsequently inoculated with SRBC, was reduced to 1/7 that for normal mice. The mice were also immunized with 10^9 SRBC after the 2nd treatment of plasmacytoma induction. Their PFC response was greately depressed. The number of PFC in these spleen cells were only 1/20 of those in normal animals of the same age.

Experiments were carried out at the level of stem cells. Bone marrow cells obtained from mice which were treated two times with the tumor induction, were transfered into X-irradiated syngenic recipients with normal thymocytes from young syngenic mice. They received the same immunization of SRBC as above mentioned, and the PFC were assayed. The intensity of their PFC response compared with that of normal bone marrow cell transfer was significantly depressed. From these results it can be suggested that immunological capability of mice after treatment of plasma cell tumor induction is significantly depressed.

It is well known that SRBC is a T-dependent agtigen. We previously reported that T cell disfunction was observed during progress of plasma cell tumor in BALB/c mice. It may be suggested that the depression of PFC response against SRBC is due to a decline of T cell function. Recovery of immune response by transfer of young thymocytes to aged mice has been reported by several investigators. The mice were injected twice with BGG-FCA, transferred of thymocytes from normal young mice, and then immunized with SRBC. The increase in PFC response in those mice was observed. The PFC level was 3.2 times higher than that of non-transferred mice. Furthermore, spleen cells obtained from BGG-FCA injected mice were transferred with normal young thymocytes into X-ray irradiated syngeneic mice. Again increase of PFC response was found, when it was compared with those obtained from mice transferred the above spleen cells only. From these results, it may be suggested that marked depression of B cell function against SRBC on the developmental process of plasma cell tumor seems to be a false phenomenon. Decrease of PFC may be due to depression of T cell function.

Recently, we found the following evidence: Aged mice, which were treated with three times of plasma cell tumor induction, were injected with *E. coli* LPS as a T-independent antigen. Seven days after the injection, all their sera had an M component with antibody activity against *E. coli* LPS, revealing B cell function of these mice was not suppressed. Furthermore, results of quantitative precipitin reaction showed that their M component was about equal amount of those from normal aged mice.

(13) Strain diffrence of five classes of immunoglobulin in inbred mice.

S. Natsuume-Sakai, K. Motonishi and S. Migita

Genetic control of the immune response to synthetic antigens

has been extensively studied in mice and guinea pig. IR gene controlling magnitude of the response is known to link with the H-2 locus in mice. Various immune disorders have been observed in several mouse strains, such as plasmacytoma of BALB/c and NZB induced by mineral oil, spontaneously developed macroglobulinemia of NZB mice, plasma cell leukemia of (DBA/2 × CBA)F_1, IgG monoclonal immunoglobulinemia of old C57BL mice and of SJL reticulum cell sarcoma. A major immunoglobulin class of the M component in those disorders has been also reported to be specific to the mouse strain.

For example, 60% of plasma cell tumors induced in BALB/c mice produce IgA, most found in (DBA/2 × CBA)F_1 and SJL disease are of the IgG_1 class, and 60% of the M component found in aged C57BL are IgG_2 class. Before the development of the disorders, we found that the level of the immunoglobulin class to which the M component belongs became higher than that of other classes in those strains.

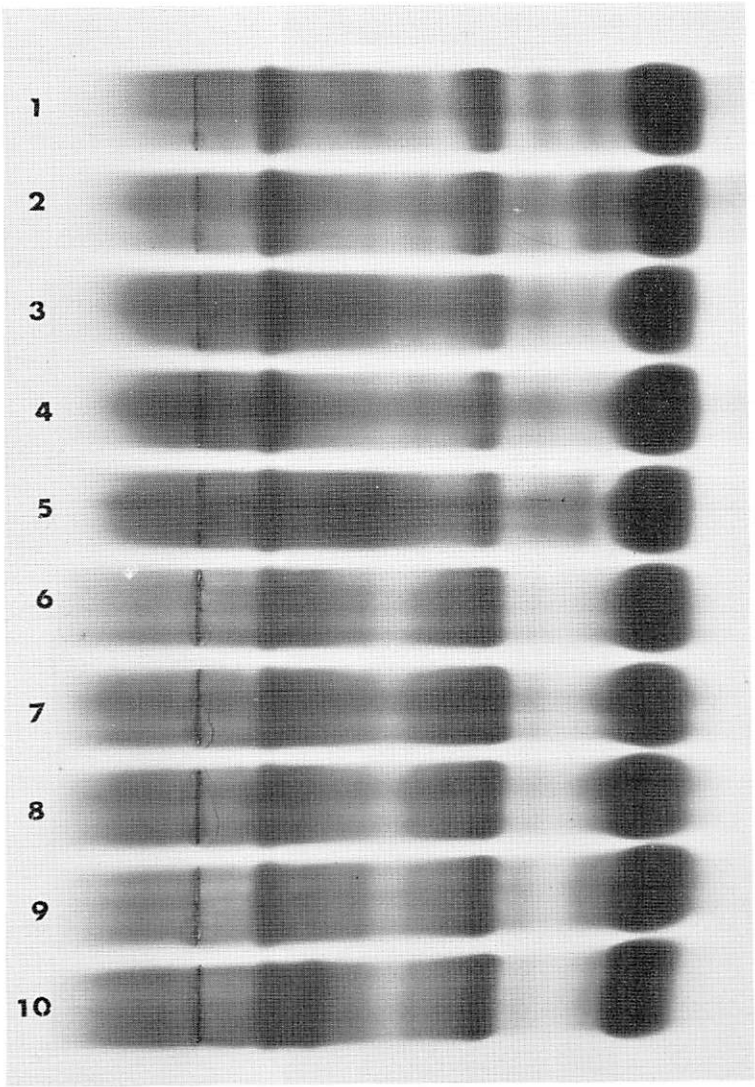
Age dependent change of the five classes of immunoglobulin level in sera of several mouse strains including BALB/c, C3H/He and C57BL/6 was investigated: The simple comparison of stained pattern by electrophoresis on agargel suggested that immunoglobulin of normal C57BL/6 mice, migrated slower in the γ region, than that of BALB/c mice. Similar results were obtained by using sera from hyperimmunized animals. For further clarification of the strain specific expression of immunoglobulin, the age dependent change of the levels of five classes of immunoglobulin in mouse serum was investigated. The concentration of each class of immunoglobulin was determined by a single radial immunodiffusion, in which rabbit monospecific antisera to mouse immunoglobulin class or subclass were employed. Although the level of all classes of immunoglobulin increased rapidly during the 3 months after birth, the increasing pattern of each class was specific to each strain. In BALB/c mice, the levels of five classes of immunoglobulin rapidly increased until the age of 8 months, whereas the extent of increase seemed to be different among the classes. The level of IgG_1, IgG_{2a} and IgA increased rapidly and exceeded 1000 μg /ml at the age of 8 months. While the level of IgM and IgG_{2b} increased during the first 2 months, and afterwards it was maintained below 500 μg/ml. After 8 months, the level of all immunoglobulin went down.

The results indicated that a major IgG subclass of BALB/c was IgG_1.

In C57BL/6 mice, the age dependent change of immunoglobulin is different from that of BALB/c mice. In all classes, the immunoglobulins increased at a slow and steady rate through the aging process. After the age of 3 months, the level of IgG_{2b} was consistently higher than any of the other subclasses ($p < 0.001$).

The levels of IgG_1 and IgG_{2a} in this strain were not significantly changed at various ages. In contrast to BALB/c mice, the levels of any immunoglobulin classes did not exceed 1000μg/ml within 12 months. Although the IgG_{2b} level in BALB/c

mice ranged from 100 to 300 μg/ml throughout the year after birth, that of C57BL/6 increased and reached as high as 700 μg/ml at the age of 20 months.



Agarelectrophoresis of sera of normal BALB/c and normal C57BL/6 mice.
Agarelectrophoresis was carried out on 1.4% agarrose in 0.05 M Tricine veronal buffer pH 8.6, at 150 V for 80 min and then fixed with 5% trichloroacetic acid in 50% methanol, dried and stained with Coomassie-Brilliant Blue G250. Anode is right.
with Coomassie-Brilliant Blue G250. Anode is right.
No. 1-No. 5 : Individuality of normal BALB/c mice.
No. 6-No. 10 : Individuality of normal C57BL/6 mice. Immunoglobulin of γ1 mobility shown near starting point is thicker in BALB/c sera than that of C57BL/6 sera. Cathodal end of immunoglobulin is longer in C57BL/6 sera than that of BALB/c sera.

In C3H/He, level of IgA, IgM and IgG_{2b} classes reached levels as high as adult mice in the age of 2 months, however, IgG_1 and IgG_{2a} levels increased rapidly at the age between 2 and 3 months. After the age of 3 months, four classes of immunoglobulin except IgG_1 changed only a little, the level of IgG_1 began to decrease until at the age of 9 months. Major IgG subclasses in this strain appeared to be IgG_1 at the age of 3 months, but the level of this class became lower than that of IgG_{2a} after 6 months, and after that time IgG_{2a} was the major class. IgG and IgA levels ranged between 200μg and 300μg ml throughout all the times measured.

From the results of three strains of mice, the following conclusion may be proposed. IgG_1, a major IgG subclass in BALB/c, is significantly higher than that of the C57BL/6 mice at any age ($p < 0.001$) under normal conventional circumstances. On the other hand, the level of IgG_{2b} is always higher in C57BL/6 than that of other strains. The level of IgA is the highest in BALB/c among the three strains. Significant differences in the level of IgG_{2a} were not observed among mouse strains.

This difference was the same in the sera from hyperimmunized or germ free animals, though the level of total immunoglobulin was different by each condition. These findings suggest that the expression of genes determing the level of the immunoglobulin classes or subclasses may be specific among the mouse strains.

(14) **Formation of a restricted antibody against E. coli lypopolysaccharide(LPS)in inbred mice: Evidence for strain differences in monoclonal antidody formation and for thymus independence.**
S. Natsuume-Sakai, O. Daimaru and S. Migita

Streptococcal group A carbohydrates and pneumococcal type III polysaccharides have the property of eliciting the homogeneous IgG antibody in the rabbit. Selective breeding studies of such immune responses in the rabbit revealed that the occurrence of homogeneous antibodies to streptococcal group A and C antigens is under genetic control. These findings of restricted antibody formation have contributed to the research of differentiation of the B-lymphocyte clone, affinity of antibody molecules to antigen and selective expression of variable region genes of the antibody molecule. We present evidence of strain differences of monoclonal antibody formation against E. coli-LPS and clear cut specificity of antibody to *E. coli*-LPS.

LPS of different lots were purchased from Difco Labolatories.

Mice were injected intraperitoneally of 50 μg LPS, following this at 7 day interval 2 more times each with 100 μg LPS. The mice were bled from the orbital plexus repeatedly at 7 day interval after the first injection. Serum specimens were investigated for the presence of homogeneous immunoglobulin.

All BALB/c mice injected with LPS from *E. coli* 055 B5 produced M component in their sera, which migrated to the slowest γ region.

Electrophoretic mobility of the M component was at the same position in all of mouse, but the other mice injected with LPS from 3 lots of Salmonella, or 3 lots of *E. coli* except 055 B5 did not produce M component in their sera. A rabbit antiserum specific for the mouse IgG_2a formed a precipitin line of M-bow, whereas other antisera specific for the mouse immunoglobulin class or subclass did not. The concentration of M components in their sera became higher after repeated injections of *E. coli* 055 B5 LPS than those obtained after primary injection.

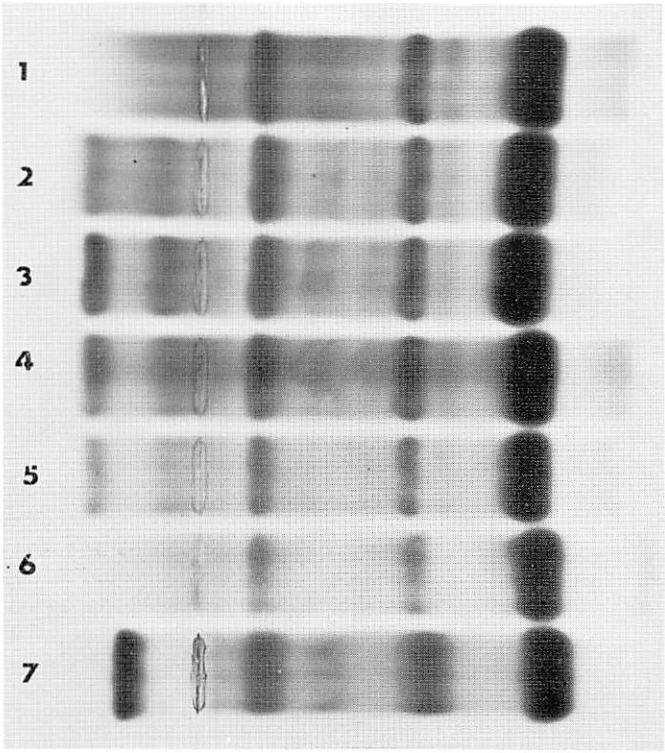
When BALB/c mice having no M component after previous injection of Salmonella-LPS were injected with *E. coli* 055 B5 LPS, M component appeared in the sera 7 days after the injection. A typical picture is shown in Fig 1. A typical quantitative precipitin curve with a symmetric figure was obtained when sera containing M component were incubated with *E. coli* 055 B5 LPS showing maximal precipitate with 50μg LPS and 10μl BALB/c serum. Other lots of LPS did not precipitate with this BALB/c serum.

It is well known that LPS is a T-independent antigen. We investigated whether or not the appearence of the M component is T-independent. Nude mice (Thymus less) of BALB/c background were intraperitoneally injected with 50μg *E. coli* 055 B5 LPS. Four of 5 tested animals still survived 10 days after injection.

The M component in all their sera was confirmed and electrophoretic mobility of M component was in the same position as that of conventional BALB/c sera. It is clear that production of M component by the injection of *E. coli* 055 B5 LPS is a T-independent phenomenon. It has been suggested that a single gene was responsible for influencing both mitogenic (polyclonal) and immunogenic (specific) responses to LPS. We carried out the analysis of strain differences in production of the M component by *E. coli* 055 B5 LPS. Homogeneous antibody in γ region was clearly detected in DBA/2, 129, A/J and BALB/c mice, but not found in C3H/He, CBA, AKR, NZW, and C57BL/6 mice. These strain differences clearly indicated that the gene for controlling restricted antibody formation against *E. coli* 055 B5 LPS is not linked to the H-2 locus, because DBA/2, BALB/c and NZW share the same H-2d haplotype, 129 and C57BL/6 also share the same H-2b haplotype. Analysis of number of gene controlling homogeneous antibodies in our system was initially carried out with (DBA/2 × C57BL/6)F_1 mice (BDF_1) and (BALB/c × C57BL/6)F_1 mice (BCF_1). Eight of the 10 BDF_1 and all of the 10 BCF_1 survived after the primary injection. All BDF_1 showed M component in their sera which migrated to the same region as that of DBA/2, and all BCF_1 showed M component in the same region as that of BALB/c. This finding suggests that dominant gene may control restricted antibody formation against *E. coli* 055 B5 LPS in BALB/c, DBA/2, A/J and 129 strain, and within inbred mouse differences exist in the variable region of specific antibodies. From results obtained with nude mice it can be suggested that IgG antibody formation is not necessary for the presence of T-lymphocyte.

The preexistence of a dominant clone of IgG restricted antibody against *E. coli* 055 B5 LPS can also be demonstrated.

Further analysis of the above mentioned points are in progress.



Agarelectrophoresis of sera obtained from BALB/c mice injected of *E. coli* 055 B5 LPS 50μg

Agarelectrophoresis was carried out on 1.4% agarose in 0.05 M Tricine veronal buffer pH. 8.6, at 150 V for 80 min and then fixed with 5% trichloroacetic acid in 50% methanol, dried and stained with Coomassie-Brilliant Blue G250. Anode is right.

No. 1 : Normal serun. No. 2 : serum obtained from mice at 7 day after the 1st injection. No. 3 : serum after the 3rd injection. No. 4 : serum after the 2nd injection. No. 5 : serum of No. 4 diluted to 1/2 with saline. No. 6 : 10 μl serum of No. 4 absorbed with 10 μl (10 μg) *E. coli* 055 B5 LPS. No. 7 : serum obtained from plasmacytoma MOPC 31C (IgG_1) bearing BALB/c mice at 21 day after the transplantation.

(15) Studies on the properties of a streptococcal preparation OK-432 (NSC-B116209) as an immunopotentiator.

I. Activation of serum complement components and peritoneal exudate cells by Group A streptococcus.

II. Tumor growth inhibitory factors elicited from human lymphocytes cultured with group A streptococcus preparation.

S. Natsuume-Sakai, Kazuo Ryoyama, Saburo Koshimura and S. Migita

It is well known that the administration of *Baccillus Calmette Guerin* (BCG) or some strains of Corynebacterium may inhibit

the growth of transplanted isogenic tumors. These organisms are not directly toxic to tumor cells in vitro, but stimulate non-specific effector function in vivo when injected into animals.

However, living Group A streptococcus exhibits a direct cytotoxic effect on tumor cells in vitro as well as in vivo, and the streptococcal preparation, OK-432, brings about beneficial effects on cancer patients. Although it is known that the anti-cancer effect of live Group A streptococcus and OK-432 is partly due to activation of the reticuloendotherial system in the host, the mechanisms of these agents have not been fully clarified.

Nishioka *et al.* have reported that microorganisms carring antitumor activity were also effective in activating the complement system. Recently, evidence has been presented that activated complement components might stimulate B-lymphocyte, leading to enhanced plaque forming cell activity against sheep red blood cells.

We are investigating the effects of Group A streptococcus (Su-strain), which is the parental strain of OK-432, on the activation of serum complement components and peritoneal exudate cells.

1) Activation of serum complement components by heat-killed (50C, 30 min) group A streptococcus (HSu-coccus).

When the C3 component or C3 proactivator is activated, electrophoretic mobility of these proteins change as follow: native C3 migrates on electrophoresis as β1C globulin, while the major fragment (C3b) produced as a result of C3 activation moves faster than β1C, as β1A. The C3 proactivator of the β-region on agarose electrophoresis shifts to the γ-region when C3 proactivator is activated. One ml of fresh human serum was added to 0.1 ml of bacterial suspension (300 μg-50 μg), and the mixture was incubated at 37C for one hour. The supernatant obtained from these mixtures were submitted to immunoelectrophoretic analysis with rabbit anti-human C3 proactivator(RAHuC3PA) and rabbit anti-human β1C β1A (RAHuC3). Fig 1 shows conversions of C3 and C3 proactivator, respectively. The immunoprecipitin line of the incubated serum against RAHuC3PA was seen in the γ-region, whereas the control serum incubated with saline the line appeared in the β-region. A partial conversion of C3 was also observed as a precipitin line of β1A mobility in human serum incubated with HSu-coccus, but only C3 of β2 mobility in the control serum. It was thus demonstrated that HSu-coccus activates the human serum complement system. The extent of activation is propotional to the amount of HSu-coccus added.

Conversion of C3 proactivator was also observed when human serum was incubated with either BCG or Group C streptococcus, although their activation seems to be less extensive than by HSu-coccus. The activation of serum complement by HSu-coccus might have occurred via an alternative pathway, because EDTA inhibited the activation but EGTA did not.

2) Characterization of peritoneal exudate cells from mice injected with HSu-coccus.

The peritoneal exudate cells were obtained from BALB/c mice injected intraperitoneally with HSu-coccus and were examined cytologically. Seven days after intraperitoneal injection of 300μg HSu-coccus, 90% of the peritoneal cells obtained were lymphocytes, and 70% of the lymphocytes were susceptible to rabbit anti-mouse thymus cell serum or AKR anti- C3H serum, using the cytotoxicity test with trypan blue dye exclusion method. The sensitivity of peritoneal lymphocytes induced by HSu-coccus to rabbit anti-mouse B-lymphocyte serum was very low. From these results, it appears that about 70% of the peritoneal cells are T-derived lymphocytes.

In control mice injected with thioglycollate, 90% of peritoneal cells were identified as macrophage on that day.

The peritoneal cells from the mice injected with HSu-coccus also responded to PHA-P (T cell mitogen), but those from normal mice did not. This also supports the finding that most of the peritoneal lymphocytes induced by HSu-coccus are T-derived.

The peritoneal cells harvested 7 days after the injection of HSu-coccus were tested for their anti-tumor activity. Mouse leukemia L1210 cells (1×10^6) were incubated at 37C for one hour with the peritoneal cells (1×10^6 - 1×10^8), and then inoculated into BALB/c mice. The peritoneal cells from mice injected with HSu-coccus completely inhibited the *in vivo* development of tumor cells, when the tumor was inoculated with the stimulated peritoneal cells in the ratio of 100 peritoneal cells to 1 (one) L1210 cell.

Even if the ratio was reduced to 10 : 1, 3 out of 5 mice survived up to 50 days. On the other hand, the peritoneal cells from normal mice did not suppress the tumor growth, even in the ratio of 100 : 1. Additionally, when the HSu-coccus was intraperitoneally injected into mice 7 days before intraperitoneal transplantation of mouse plasmacytoma MOPC 31C, there was no sign of tumor growth, at least up to 90 days in all experimental animals.

This evidence suggests that antitumor effects of peritoneal lymphocytes obtained from the mice injected with HSu-coccus are nonspecific, since L1210 and MOPC 31C do not share the common tumor antigens. It may be possible that the anti-tumor activity of OK-432 is mediated by soluble factors from lymphocytes rather than by direct cell to cell interaction.

We could not determine whether or not complement components activated by HSu-coccus play a role in stimulation of immunocompetent cells. But it is well known that B lymphocytes and macrophage have C3 receptors on their cell membrane, and the activated C3 component releases chemotactic factors.

Recently, we obtained data that the level of serum IgM and IgG_2 in BALB/c, C57BL/6 and BDF_1 mice increases after the administration of OK-432. Furthermore, our preliminary experiments showed that HSu-coccus directly activated lymphocytes. When normal human lymphocytes were cultured with HSu-coccus at 37C for 72 hours, incorporation of ^{3}H-thymidine in them was significantly higher than that of a control culture in-

cubated with saline. The supernatant from the mixed culture of HSu-coccus and human lymphocytes inhibited the growth of L1210 and MOPC 31C. From these data, it may be suggested that human lymphocytes activated by HSu-coccus release soluble factor of antitumor activity.

Results of characterization of this factor showed it to be non-dialysable, stable after treatment at 56C for 30 min, but labile at 100C. Further analysis of this factor is in progress.

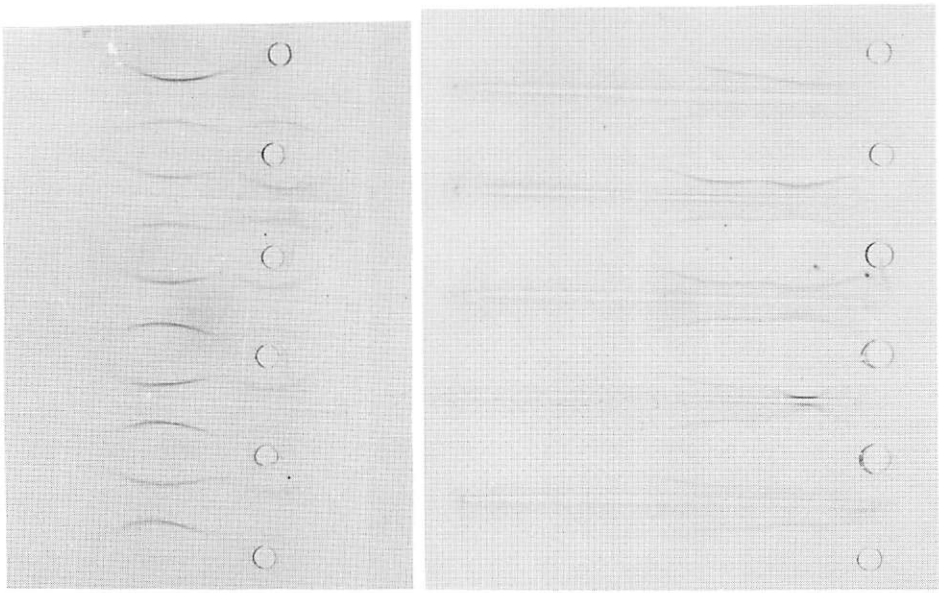


Fig. 1-a. Immunoelectrophoretic patterns of C3 proactivator in human serum incubated with HSu-coccus.

Immunoelectrophoresis was carried out using 1.4% Dojine Agarose II in veronal buffer (pH 8.6, μ=0.005). The left is anode. Precipitin reactions were developed against rabbit anti human C3 proactivator. Each of antigen wells contain 5 μl of test material which consists of 1 *ml* of human serum and 0.1 *ml* of bacterial suspension or 0.85% saline preincubated at 37C for an hour. Antigen was filled from top to bottom, in the order of saline, 300 μg HSu-coccus, 200 μg HSu-coccus, 100 μg HSu-coccus, 50 μg HSu-coccus and saline.

Fig. 1-b. Immunoelectrophoretic patterns of C3 component in human serum incubated with HSu-coccus, BCG or Group C streptococcus.

Immunoelectrophoresis was carried out as mentioned in Fig. 1-a. Precipitin reactions were developed against rabbit anti human C3 serum. Antigen was filled from top to bottom: Saline, 300 μg BCG, 300 μg HSu-coccus, 200 μg HSu-coccus, 100 μg HSu-coccus, 300 μg Group C coccus.

(16) A device for preserving standard lyophilized serum for protein analysis has revealed minimal changes in 30 components over one year.

K. Hirohashi*, D. Inoue* and S. Migita

Standard serum is essential for protein and enzyme analysis as a stable control in clinical examination. However, serum contains many fractions that differ in chemical and physical properties from each other. Some fractions may be denatured easily, while others are stable. It is difficult to preserve all

serum fractions naturally for at least one year. We have attempted to show that lyophilized serum with preservatives may be used as a standard serum, especially for the control sample of agar electrophoresis, immunoelectrophoresis and single radial immunodiffusion.

Since a small amount of the standard serum is enough for a single analysis, pooled normal serum was divided into each 50μl of a plastic hole plate, was lyophilized and was stored in the cold. Whenever we needed control serum, one hole was cut with scissors, 50μl of distilled water was added and the reconstituted serum was used as standard.

1. Volume change of reconstruction: When 1ml of serum was lyophilized and 1ml of water was added, we got 1.07ml of reconstituted serum. When 1.07ml of serum was lyophilized and dissolved in 1.0ml of water, 1.069ml of serum was obtained.

2. Preservatives for lyophilized serum: Ethylendiamine tetraacetic acid, sodium azide and glycine were added to the pooled serum as preservatives to the final concentation of 0.1M, 0.5%, 1M, respectively. EDTA prevents conversion of C1s to C1$\bar{s}$ that mediates the classical pathway of the complement system. Sodium azide prevents bacterial growth and enzyme activity. Glycin is for good solubility of the lyophilized serum. Actually 53ml of pooled serum was added to 47ml of 0.2M EDTA, 1% sodium azide and 2M glycin, then each 100μl was divided into the hole plate for 53μl of original serum.

3. Solvent of the preservative: Distilled water and saline were compared as solvent for the preservative. Though the solvent is evaporated by lyophilization, less denaturation of albumin was confirmed by agar electrophoresis when saline was used as solvent than for the case of distilled water.

4. Container for lyophilized serum: Glass vial and hole plate of poly-vinyl chloride were compared as containers for lyophilized serum. The glass vial looks better for the container at first, but when the hole plate made by a special sandwich plate constructed with 2 sheets of poly-vinly chroride and one of polyethylene was used, the lyophilized serum could be kept without denaturation for a longer period. In a later experiment, the hole plate was put into a second air tight container of cylindridal form made of polyvinyl chloride, with silica gel as dehydrizer.

5. Source of the serum: A large amount of fresh and pooled serum of many normal individuals are needed for the source of standard serum. All of the spare serum from the blood donor in a Blood Transfusion Bank was placed in -80C. Positive sera of hepatitis B antigen or Wasserman reaction were omitted.

The accumulated amount for 1 week was 1 to 2 l from 500 to 800 individuals and was pooled as 1 lot.

6. Qualitative tests on new standard serum kept at different temperatures: The lyophilized serum in single or double containers were placed in -80C. -20C, +4C, +20C, +37C for 1 week, 3 weeks, 3 months and 12 months respectively. One hole of the plate was cut, mixed with 50μl of distilled water, and then the samples were compared with each other by agar electrophoresis, immunoelectrophoresis with monospecific antiserum to

each fraction as well as with antiwhole-human-serum. β-Lipopretein, ceruloplasmin, C3, C3 proactivator and β2 glycoprotein II were denaturated, showing changes in their mobility, when the containers were kept at higher temperature than 4C and for longer than 3 week.

However, if it was kept under -20C, all fractions except albumin looked unchanged so far as we examined. Polymerization of albumin was confirmed by the elongated precipitation line by immunoelectrophoresis. Denaturation of serum fractions were reduced when we employed a hole plate of sandwich type layer and a double package.

	mean	SD		mean	SD
Pre	94%	22%	ATIII	104%	28%
alb	100	15	Hx	112	26
α1AT	111	25	β2III	100	18
α1AG	103	25	Tf	107	29
α1B	108	24	βL	121	32
α1X	97	26	C4	102	31
Iα1TI	103	25	Pmg	103	22
Znα2	99	28	C3	108	25
Hp	93	27	C3PA	93	29
Cp	108	26	β2I	100	25
α2M	125 (113	42 23)*	IgM	116 (106	52 35)*
α2HS	104	24	IgG	101	21
α1sI	108	28	IgA	94	34
C9	102	34	IgD	104	51
C5	101	23	IgG3	113	66

Tab. 1 : Normal value (mean and standard deviation of 95 normal individuals from 2 to 65 years of age) of 30 serum components expressed percentage of a pooled lyophilized normal standard serum as 100.

* mean and SD obtained from 83 adult normal individuals over 20 years old.

7. Normal individual value estimated by this standard serum as control: Each 30 fractions of 95 normal individual sera were estimated by micro single radial immunodiffusion with this standard serum as control. The value expressed by percentage using the standard serum as 100. The results obtained had a

good mean value between 93 and 115 and showed a relatively narrow standard deviation within 30% in 23 components, except α2M, C9, C4, βL, IgM, IgA and IgD. (Tab. 1)

This indicates that our control serum can be used and preserved as a standard of serum protein analysis for at least one year.

---

* Ogata Institute for Medical and Chemical Research

(17) Studies on the basic mechanism of single radial immunodiffusion.
S. Migita and S. Ohgaki*

Single radial immunodiffusion (SRID) has been widely employed for quantitative estimation of antigens or antibodies since Mancini and Fahey reported separately in 1965. The basic mechanism of this method was discussed by Becker or Heremans. Antibody titer, mg of antigen precipitated per ml of the corresponding antiserum, can be obtained from the antigen applied and the amount of antiserum present in the precipitin ring according to Becker [1]. Such antibody titer has been written on the bottle of antisera commercially available in every country. We found an error in the basic mechanism of SRID and in the estimation of antibody titer described by Becker.

1. Participation of antibody from the outer area of the precipitin ring.

The name single radial immunodiffusion means that only the antigen diffuses radially into the gel and the antibody does not move during the immunoreaction, because the antibody is located homogeneously in the gel at the beginning. However, that the density of precipitin ring is thicker on the outline of the ring indicates a heavier antigen-antibody complex located in the outline than in the center of the ring. We can not explain unequal distribution of density in the precipitin ring unless antibody from the outer area gathers and participates in the reaction.

If antibody does not move during the reaction, we can expect the same concentration of antibody as before in the outer area even after the first SRID was performed. When the 2nd SRID was done in the outer area of the 1st SRID of the same plate, this was proved wrong because a larger precipitin ring was produced with the same amount of antigen to the 1st SRID.

When the SRID reaction was stopped in the middle of diffusion by fixation with ethanol and acetic acid, the stained plate with amido black 10B revealed that the antigen was heavier near the antigen well when the diffusion was continuing, but heavier in outline and lighter in the center when the diffusion was completed. This indicated the basic mechanism of SRID to be as follows: 1) The antigen diffused into the antibody agar gel and the antigen-antibody complex was produced at the equivalence point. 2) Then more antigen arrived from the well to the precipitate, the complex was solubilized by the excess antigen. 3) The solubilized complex diffused into the outer area and changed to precipitate again by the additional binding of anti-

body. 4) Such a process continued until the antigen well became empty and the soluble complex of antigen excess changed to insoluble complex in an equvalent ratio. 5) Free antibody located in the outer area of the precipitate diffused in the reverse direction to that of antigen and a heavier precipitate was produced on the outer part of the precipitin ring.

2. Ratio of antibody participating to precipitation from inside and outside of the precipitin ring.

The ratio of the antibody participating to precipitation from inside and outside of the precipitin ring was calculated by several methods. 1) The maximum diameter of precipitation was almost constant, when a commercial SRID plate for human immunoglobulin classes was used. If more antigen than this was applied in the well, the final precipitin ring was obscure. This indicates that there is no antibody in the outer area of maximum precipitation.

The ratio of the area of the precipitin ring and that of the outer space correspond to the amount of antibody. 2) Isotopic count for the estimation. When ^{59}Fe labeled human transferrin was mixed with agarose and the well was filled with antisera, the isotopic count of the precipitin ring was 2.7 times greater than that expected from the area of the precipitin ring and original density of ^{59}Fe. 3) Antibody titer obtained by quantitative precipitin curve. Antibody titer of goat anti human-IgG was obtained by quantitative precipitin curve or by SRID. Antibody titer of SRID was 3 times greater than that of the quantitative precipitin curve. These results indicate the ratio of antibody participating to the precipitation from inside and outside of the precipitation ring is 1 : 1.8 to 2. A detail of this research will be published in 2).

Reference:

1) Becker W., Immunochemistry, 6: 539-546, 1969
2) Migita S., Rinsho-Kensa, (J. Medical Technology) 20: 259-271, 1976

---

* On leave from Dept. Medicine, Kagoshima Univ. School of Medicine.

(18) Correlative change of serum acute phase proteins and components of complement in cancer patients.

S. Migita, K. Okamoto, K. Horiuchi and M. Shako*

Single radial immunodiffusion according to Mancini was modified for microscale in our laboratory. Six holes of 1 mm in diameter were prepared in a 2 × 2.5 × 0.1 cm antibody-agarose plate. According to a protocol determined, 0.4 to 0.8μl of 1/2 or 1/8 diluted serum was put in the hole by a Hamilton microsyringe with a repeating dispenser which was fixed on a stand. Sera from 95 normal individuals, 280 different cancer patients and 130 other diseases were each measured for 30 components of serum protein with 30 specific antisera, respectively.

Thirty components were as follows, prealbumin, albumin, α1 antitrypsin (α1AT), α1 acid glycoprotein (α1AG), α1 B gly-

coprotein, α1 antichymotrypsin (α1X), inter α-trypsin inhibitor, Zinc binding α2 glycoprotein, haptoglobin (Hp), ceruloplasmin, α2 macroglobulin, α2 HS globulin, C1s inhibitor (C1sI), C9 component (C9), C5 component (C5), antithombin III, hemopexin, β2 glycoprotein III, transferrin, β lipoprotein, C4 component (C4), plasminogen, C3 component (C3), C3 proactivator (C3PA), β2 glycoprotein I, immunoglobulin M, immunoglobulin G, immunoglobulin A, immunoglobulin D and immunoglobulin G3. Lyophilized normal pooled serum in a microcup was used for a standard of the quantification as 100%.

Concentration of each fraction in individual serum was expressed by percentage of the normal standard. The data obtained were analysed by a FACOM 230 electronic computer.

Increase of actue phase proteins in cancer patients is well known; however, which fractions of the serum belong to acute phase protein has not yet been established. From statistical analysis of the 280 cancer sera, we estimated α1AT, α1AG, α1X and Hp as acute phase proteins, because of the high correlation coefficient among them. Significant increas of the all 4 components over 2 times of normal standard deviation was confirmed in cases of peritoneal or lung metastasis of cancer. They showed a medium increase of over 1SD in cases of cancer of stomach, lung, intestine and pancreas. This suggests that activated macrophage may produce stimulating factors and the factors may stimulate the liver for increased synthesis of 4 acute phase proteins. However, all 4 components do not equally increase in cases of acute myeloblastic leukemia (AML) and cancer of prostata, but only α1AG, α1X and Hp increase in AML and α1AT increases in cancer of prostata. This suggests that reticulum cells of bone marrow may secrete a stimulating factor for α1AT, because cancer of prostata are closely related to the bone marrow and AML is the disease of bone marrow. Consequently the macrophage of other tissues may be related to the stimulated rate of α1AG, α1X and Hp production.

Another character of cancer sera was the increase of components of complement, C3, C4, C5, C9, C1sI and C3PA, though these components did not increase equally. Since activated components of complement work as chemotactic factors(C5a, C567), anaphlatoxin (C3a, C5a), the key of B cell differentiation (C3b), and α1AT and α1X work as proteinase inhibitors, we thought that their increase were advantage for his own nonspecific defense to cancers.

Since every component has different functions for protection of human existence and the increase occured unevenly in these components, we thought that the meaning was his own multiple protection against every disease from which we may suffer. Correlation coefficients between acute phase proteins and complement components were obtained by electronic computer in different groups of cancer in order to find the maxiaml correlation. As shown in Tab. 1, good correlation was found between the mean value of acute phase proteins and the mean value of C1sI and C4 in normal individuals, between the mean of acute phase proteins and the mean of C5, C9 and C3PA in AML or

cancer of the stomach and between the mean of acute phase proteins and above both means of complements in cancer of the lung or intestine.

This suggests that mobilization of acute phase proteins and components of the classical pathway occured in the normal case.

Acute phase proteins and those of alternate pathways mobilized in cancer of the stomach or AML. Acute phase proteins and complements of both pathway increased in cancer of the lung or intestine.

The mobilization of these depended upon the size and stage of the tumor, metastasis and therapy performed. The increase of these components was also confirmed after immunotherapy of AML with OK432, streptococcus A product, and the decrease was significant after complete surgical extripation of cancer of the stomach or kindney. These suggest that whether acute phase proteins and components of complement have increased in the sera or not is a key for the indication of immuno-therapy to be perfermed for cancer patients, because their increase indicated that the mechanism of the non-specific defense was already mobilized and the space of the mobilization in the future by immunotherpy might be limited.

---

* Data Processing Center, Kanazawa University

	Cases	C4	C3,C4	C3,C5	C5,C9	C3PA,C5,C9
normal	97	0.44	0.50	0.28	0.13	0.27
C. stomach	48	0.18	0.31	0.37	0.57	0.54
AML	28	0.06	0.05	0.11	0.79	0.75
C. lung	11	0.61	0.73	0.32	0.63	0.79
C. intestine	15	0.55	0.54	0.35	0.86	0.74
C. kidney	25	0.29	0.41	0.36	0.71	0.63

Tab. 1 : Correlation coefficients between the mean concentration of 4 acute phase proteins and the mean concentration of components of complement. Combinations of components of complement were selected in order to find the maximum correlation coefficient.

(19) The alteration of cell surface antigenicity of the mouse plasmacytoma. Lack of correlation between the synthesis of myeloma protein and the alteration of surface antigens.

S. Ohno, S. Natsuume-Sakai and S. Migita

The correlation between the change of immunoglobulin synthesis and the surface antigenicity was analysed by the cytotoxicity and quantitative antibody-absorptin tests with the cells of immunoglobulin-producing and nonproducing mouse plasmacytoma. IgA-synthesizing BALB/c plasmacytoma 58-8 and

non-IgA-synthesizing variant of the 58-8 (nonproducer) were equally killed with anti-58-8, anti-H-2d and anti-PC.1 antiserum plus complement, respectively, only when the cells were pretreated with pronase. Quantitative absorption tests revaled that the nonproducer 58-8 has had much the same amount of the plasmacytoma antigen of 58-8, PC.1 antigen and a greater amount of H-2d antigen as producer 58-8. Same subject was also analysed for the C3H mouse plasmacytoma X5563 which has a M-component of IgG2a. The nonproducer X5563 has expressed a greater amount of H-2k antigen and a smaller amount of the plasmacytoma antigen of X5563 than the producer X5563. No detectable PC.1 antigen was observed at surfaces of the producer and the nonproducer X5563, respectively.

(20) **In vitro proliferative response of BALB/c mouse spleen cells stimulated with trinitrophenylated syngeneic spleen cells**

H. Tokuyama

Mouse spleen cells can react with hapten-conjugated syngeneic spleen cells in mixed lymphocyte culture. Trinitrophenylated (TNP) spleen cells were prepared by treating normal spleen cells with sodium 2, 4, 6-trinitrobenzenesulphonate. Four-day cultures of TNP-labelled spleen cells incorporated 2.5-7.4 times more ^{3}H-thymidine than similar cultures of untreated spleen cells.

An obviously positive mixed lymphocyte reaction (MLR) by normal spleen cells against mitomycin C (MC) treated TNP-labelled syngeneic cells was observed after 4 days of culture. The MLR to TNP-labelled syngeneic cells were inhibited in the presence of ε-TNP-L-Lysine by 23-37%. The spleen cells from the mice injected intraperitoneally with TNP-labelled syngeneic spleen cells showed a higher MLR against TNP-labelled spleen cells than did normal spleen cells. The sensitized spleen cells also showed an increased response to MC-treated spleen cells. These results suggest that normal spleen cells include cells which can recognize the hapten and new antigenic determinants introduced into syngeneic spleen cells by chemical modification.

Immunology, 29, 875-884, 1975.

(21) **Solubilization of immune complex by complement activated by the alternate pathway but not by the classical pathway**

M. Takahashi, V. Brade* and V. Nussenzweig**

Solubilization of precipitated immune complexes by C' (CRA) (Miller and Nussenzweing, PNAS *72*, 418, 1975) was studied using radiolabelled BSA-rabbit anti-BSA and human or rabbit C'. Properdin (P) and factor D (D) were found to be essential for CRA as well as C3, factor B and Mg^{++}, as reported earlier. Addition of a physiological concentration of purified $\overline{P}$ or $\overline{D}$ to either P-depleted serum or D-depleted serum completely restored CRA.D-depleted serum contains unreduced levels of classical pathway components C1, C4, C2, C3, but failed to induce solubilization of immune complexes. This indicates, therefore,

that classical pathway alone cannot induce efficient CRA. However, the classical pathway, along with the alternate pathway, greatly enhances this reaction, as previously reported. Sucrose density gradient ultracentrifugation of solubilized complexes revealed that CRA involved both disaggregation of large aggregates into smaller aggregates, and dissociation of antibody from immune complexes. It appears that the incorporation of C' components into critical regions of the antigen-antibody lattice favors the dissociation of antibody and causes solubilization.

---

* Inst. Med. Mikrobiol., Univ. Mainz, Germany
** New York University, School of Medicine, New York, U. S. A.

(22) **Inefficiency of the terminal component of guinea pig complement (C9) in the lysis of guinea pig erythrocytes: Binding of C9 without lysis**

K. Yamamoto and M. Takahashi

Using the reactive lysis system, a form of hemolysis mediated solely by late-acting complement components, C5-C9, guinea pig C9 was found very inefficient in inducing lysis of isologous and mouse erythrocytes which had reacted with C5-C8. Guinea pig C9 was shown to effectively lyse C5-C8 reacted sheep or goat erythrocytes and human C9 could lyse equally well C5-C8 reacted erythrocytes from all of the above mentioned species. Further studies showed that guinea pig C9 was able to bind to C5-C8 reacted guinea pig erythrocytes (guinea pig EC5-8) and rendered them resistent to the subsequent lytic action of human C9. Addition of excess guinea pig C9 to the mixture of guinea pig EC5-8 and human C9 effectively inhibited subsequent hemolysis. Our finding shows that guinea pig C9 is able to bind to guinea pig EC5-8 but unable to induce a subsequent lytic process on the membrane of guinea pig erythrocytes.